RESEARCH ARTICLE

Green Synthesis of Bioactive CuO@Fe₃O₄@Walnut Shell Nanocomposite Using *Crataegus azarolus* Var. *aronia* L. Extract and its Antivasoconstrictive Action on Rat's Aortic Smooth Muscle

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ABSTRACT

The antioxidant potential of *Crataegus azarolus* Var. *aronia* aqueous extract was used as reducing and stabilizing media to the green synthesis of bioactive CuO@Fe₃O₄@Walnut shell nanocomposite (NCs). The ability of the plant extract as bioreducing medium was proved using Fourier-transform infrared spectroscopy and ultraviolet-visible spectroscopy. While, the structural identification of synthesized CuO@Fe₃O₄@Walnut shell NCs was proved using the scanning electron microscopy, electron dispersive spectroscopy, elemental mapping, and point analysis. Then, the antivasoconstriction effects of CuO@Fe₃O₄@Walnut shell NCs (5*10⁻² mg/ml) and (1*10⁻¹ mg/ml) on rats isolated thoracic aortic smooth muscle cells were studied. Green synthesized CuO@Fe₃O₄@Walnut shell NCs showed effective antivasoconstriction activity against norepinephrine (NE) (1*10⁻⁹–10⁻⁴ M) induced contraction in endothelium-intact and endothelium-denuded rats aortic smooth muscle. In conclusion, the aqueous extract of *C. aronia* acts as potential reducing and stabilizing media to the green synthesis of bioactive CuO@Fe₃O₄@Walnut shell NCs and the NCs play an important role against contractile effects of NE on rats' aortic smooth muscle.

Keywords: Aortic smooth muscle; *Crataegus aronia*; Green synthesis; Nanocomposite

INTRODUCTION

Medicinal plant is referred to the use of herbs in traditional medicine for prevention and treatment of various diseases. The applications of herbal plants dated back to ancient times (Ayrle et al., 2016, Arshad et al., 2020). Plant phytochemical screening revealed the presence of various bioactive compounds with different biological actions such as alkaloids, polyphenols, essential oils, tannins, quinones, sterols, and saponins (Manzoor et al., 2016). Medicinal plants appeared as the important sources for natural medications with no side effects, availability, and low costs. Phenolic acids, ascorbic acid, tocopherols, and bioflavonoids are some plant phytochemicals having potential natural antioxidant activities and mainly used to treat various diseases (Granda and De Pascual-Te, 2018). Typical phenolic compounds that possess antioxidant activity are predominantly phenolic acids and flavonoids (Mahmud, 2017a, Mahmud, 2017b). Nearly 75–80% of the famous population of the developing country, herbs present a dominant role in the health-care system (Qazi and Molvi, 2016).

Recently, in drug discovery, the herbal products were strongly employed (Mohsenzadeh et al., 2016). Herbal products are widely used to treat numerous health problems such as joint, skin, gastric (Alvi et al., 2018), hepatic, respiratory (Varga et al., 2018, Anand et al., 2019), and heart (Ninh, 2017), atherosclerosis, hypertension, angina pectoris, congestive heart failure (Rastogi et al., 2015), and also can act as antifever, anticancer, and antiproliferation (Lim et al., 2019).

The hawthorn (*Crataegus* spp.) includes more than 200 species, distributed throughout Europe, East and Central Asia, and North America (Mahmud et al., 2016). It contains important chemicals such as phenols, flavonoids, alkaloids, steroids, terpenoids, and tannins which give the plant essential value in phytochemical and pharmaceutical view (Mirzaei and Mirzaei, 2013). The hawthorn is essential in traditional and formal medicinal sciences because of its numerous biological actions such as vasodilator, anti-inflammatory, antioxidant, positive inotropic, and cholesterol synthesis preventing properties (Liperoti et al., 2017).

Heart and blood vessel problems mostly lead to cardiovascular diseases (CVDs) such as coronary heart disease, stroke, hypertension, peripheral artery disease, rheumatic heart disease, congenital heart disease, and heart failure (Rastogi et al., 2015). Some studies have indicated that several hawthorn species are widely used for the treatment of CVDs in folk medicine (Abu-Gharbieh and Shehab, 2017). On the other hand, the previous studies (Mahmud et al., 2016, Keser et al., 2014) showed that leaves, flowers, and fruits of *Crataegus* can be used to treat CVDs and as hypotensive agents. Different dried or fresh parts of hawthorn can be used in manufacturing of teas and preparation some dosage forms of over-the-counter medicines or dietary supplements (Bekbolatova et al., 2018). Furthermore, it has been found that *Crataegus azarolus* L. in Arab herbal medicine apply for CVDs as well as diabetes mellitus and cancer (Bechkri et al., 2017). The present study investigates the effects of green synthesized bioactive CuO@Fe₃O₄@Walnut shell nanocomposite (NCs) on a contracted rat's aortic smooth muscle.

For limitations of using nanostructures in sensitive aspects of science and technology due to the accumulation of dangerous materials on their surface (Hosseinkhani et al., 2012), agglomeration process and also altering the surface of nanostructures for their high tendency to react with humidity, oxygen and some chemicals (Nemamcha and Rehspringer, 2006), the green method was employed as an efficient procedure to overcome these problems. Recently, the biological systems of nature are employed as green

factories to produce the nanomaterials through the energy saving and biocompatible paths for deposition of bioactive constituents on nanosurface (Sajadi et al., 2018a, and Sajadi et al., 2018b). The nanoparticles have an extensive surface area containing numerous active sites per unit area caused to their various applications (Sajadi et al., 2019).

Furthermore, due to the presence of a huge content of antioxidants inside *C. aronia* plant extract and also to decrease the agglomeration and aggregation of nanoparticles, both the aqueous plant extract and Walnut shell were used as a potential source of reducing phytochemicals and biotemplate to reducing the iron and copper ions and converting them to the magnetite and copper oxide nanoparticles deposited on the surface of Walnut shell as a natural substrate, Scheme 1.

MATERIALS AND METHODS

Instruments and Reagents

High-purity chemicals were purchased from Merck and Aldrich chemical companies. The morphology, particle dispersion, and chemical composition of the prepared nanostructures were investigated by FE-scanning electron microscopy (SEM) (Quanta 450) equipped with electron dispersive spectroscopy (EDS). The antivasoconstriction effects of green synthesized bioactive CuO@Fe₃O₄@Walnut shell NCs on norepinephrine (NE)-induced contraction were studied using the rat's aortic smooth muscle.

Scheme 1: Schematic biosynthesis of CuO@Magnetite@Walnut shell NCs using *Crataegus aronia* extract

Laboratory Animals

Male albino rats, *Rattus norvegicus* ranging 200–300 g, were used in the present study. Animals were housed in plastic cages bedded with wooden chips and kept under standard laboratory conditions at 22 ± 2 °C and exposed to a photoperiod of 12 h light followed by 12 h of darkness, using an automated light switching device. The rats were fed on standard rat pellets with free access to dechlorinated tap water *ad libitum* (Mahmud and Mahmud, 2013; Abdulla et al., 2017).

Preparation of Plant Extract

Fifty grams of the dried fruit of *C. aronia* were mixed to 200 mL distilled water at 85°C while stirring for 1 h. The mixture then was filtered and filtrate was used as *C. aronia* extract for further investigation.

Single Pot Biosynthesis of CuO@Magnetite@Walnut Shell NCs Using *C. aronia* Extract

In a 250 mL flask, 1 g dried powdered Walnut shell was mixed with 100 mL plant extract, then 50 mL 0.3% $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.6% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solutions were added dropwise, respectively, to the mixture in an alkaline media adjusted using 0.1 M Na_2CO_3 while stirring at 80°C until changing the color of the mixture and formation of $\text{CuO}@\text{Fe}_3\text{O}_4$ @Walnut shell NCs precipitation. The mixture then filtered and obtained precipitate was dried and kept to identification and application processes as a bioactive agent.

Preparation of Aorta and Experimental Design

The procedure presented by Al-Habib et al. (2015), was used with few modifications for preparing the rat's aortic rings and calibrating the same instrument's model to study the vascular reactivity in the isolated aorta. The antivasoconstriction effects of two doses (5×10^{-2} M and 1×10^{-1} M) of $\text{CuO}@\text{Fe}_3\text{O}_4$ @Walnut shell NCs on aortic rings post-contracted with different doses of NE (1×10^{-9} – 10^{-4} M) following an incubation period of 30 min were studied.

The current study included the following experimental groups.

Group I

To study the antivasoconstriction effects of $\text{CuO}@\text{Fe}_3\text{O}_4$ @Walnut shell (5×10^{-2} mg/ml) and (1×10^{-1} mg/ml) NCs in endothelium-intact aortic rings post-contracted with different doses of NE (1×10^{-9} – 10^{-4} M), after pre-incubation of green $\text{CuO}@\text{Fe}_3\text{O}_4$ @Walnut shell NCs for 30 min.

Group II

To elucidate the role of endothelial cells in antivasoconstriction effects of $\text{CuO}@\text{Fe}_3\text{O}_4$ @Walnut

shell (5×10^{-2} mg/ml) and (1×10^{-1} mg/ml) NCs using endothelium-denuded aortic rings post-contracted with different doses of NE (1×10^{-9} – 10^{-4} M), after careful removal of endothelium. The removal of the endothelium was confirmed by the absence of relaxation induced by ACh (1×10^{-5} M) following NE (1×10^{-6} M) pre-contraction.

Statistical Analysis

All data were expressed as means $\pm$ SEM and the median effective concentrations (EC_{50}) values are given as geometric mean with 95% confidence intervals (CI). The statistical analysis was performed using two-way analysis of variance (ANOVA) supported by Bonferroni test when carrying out a pairwise comparison between the same dose of different groups using GraphPad Prism program (Version 8.0.2) (GraphPad Software, 2019, USA). $P < 0.05$ was considered as statistically significant. In all figures the symbols, *, **, and *** represent the significance of the mean of differences at the 0.05, 0.01, and 0.001 levels, respectively.

RESULTS AND DISCUSSION

Spectrophotometric Study of the Plant Extract

The ultraviolet–visible of the plant extract shows some main signals at 285 and 345 nm assigned to the benzoyl and Cynamoeil ring of phenolic antioxidants [Figure 1]. Furthermore, the Fourier transform infrared spectra of the prepared extract demonstrated some signals at around 1120 cm^{-1} , 1420 cm^{-1} , 1690 cm^{-1} , and 3600 cm^{-1} , where are belonging to the C–O, C=C aromatic, C=O, and OH functional groups. These results strongly confirm the presence of antioxidants compounds, especially antioxidant phenolics inside the plant extract. Thus, the mentioned extract can be used as a reducing media to biosynthesis of nanostructure and stabilizing them [Figure 2].

Characterization of Biosynthesized $\text{CuO}@\text{Fe}_3\text{O}_4$ @Walnut Shell NCs

During this study, the $\text{CuO}@\text{Fe}_3\text{O}_4$ @Walnut shell NCs were fabricated using the Walnut shell dried powder and

Figure 1: Ultraviolet–visible spectrophotometer of *Crataegus aronia* fruit extract, signals at 285 and 345 nm are assigned to the benzoyl and Cynamoeil ring of phenolic antioxidants and their related chromophores

C. aronia extracts during a one pot procedure. The FE-SEM, EDS, and elemental mapping analysis were employed to further study of the morphology and structure of the NCs [Figures 3-5, respectively]. As Figure 3 shows, beside the presence of some agglomerations, the deposition of spherical and homogenous nanoparticles (50 nm–80 nm) on the substrate (Walnut shell) surface is absolutely considerable. Therefore, according to these micrographs, the fabrication of NCs is simply confirmed.

Further, the EDS spectrum and elemental mapping represent the well-defined peaks of compositional elements of NCs including Fe, O, Cu, Al, and Mg. Thereby, these results confirm the successful anchoring of the CuO and magnetite NPs on the surface of Walnut substrate [Figures 4 and 5]. Therefore, all previous analyses showed the fabrication of the CuO@Fe₃O₄@Walnut shell NCs.

Furthermore, the present study was performed to investigate the possible antivasoconstriction effects of the CuO@Fe₃O₄@Walnut shell NCs green synthesized by *C. aronia* extract on rat aortic ring smooth muscle at doses (5×10^{-2} mg/ml) and (1×10^{-1} mg/ml). Diverse physiological responses to synthesized CuO@Fe₃O₄@Walnut shell NCs may reflect the presence of various plant bioactive compounds which produce responses in the target cells

Figure 2: The Fourier transform infrared spectrum of the *Crataegus aronia* fruit extract, signals appeared at 1120 cm⁻¹, 1420 cm⁻¹, 1690 cm⁻¹, and 3600 cm⁻¹ are belonging to the C-O, C=C aromatic, C=O, and OH functional groups related to the phenolic antioxidants

Figure 3: FE scanning electron microscopy micrograph of CuO@Fe₃O₄@Walnut shell nanocomposite, as the micrographs show the size, shape, and morphology in a nanoscale dimension

through various signal transduction mechanisms. The endothelium layer of the vascular wall synthesizes and releases a broad spectrum of vasoactive substances that play a fundamental role in the regulation and maintenance of cardiovascular homeostasis (Triggle et al., 2012). Nitric oxide (NO), prostaglandin I₂ (PGI₂), and the endothelial-derived hyperpolarizing factor (EDHF) are the main endothelial-derived factors that relax vascular smooth muscles (Luna-Vazquez et al., 2013).

The current investigation revealed that endothelium and EDHFs play an important role in antivasoconstriction effects induced by CuO@Fe₃O₄@Walnut shell NCs. The inhibitory effects of green NCs on the contraction of rat aortic rings observed in the current study may be mediated by induction of endothelium relaxant factors such as NO and PGI₂ production since the application of indomethacin (non-specific cyclooxygenase inhibitor), the antivasoconstriction effect of NCs indicates that PGI₂ might play an indispensable role in antivasoconstriction effect. The PGI₂ causes relaxation of vascular smooth muscle cells through the stimulation of G-protein-coupled receptors, which, in turn, activates adenyl cyclase and thus elevates the level of cyclic adenosine monophosphate that inducing vasodilation (Luna-Vazquez et al., 2013). Green NCs induced reduction in contractions of aortic rings through the production of PGI₂ is consistent with some previous studies. Methanol extract of *Crataegus* contains several phenolic compounds with myriad biological activities modulate various physiological actions in mammals (Liu, 2012), these compounds may adsorbed on the surface of NCs. Since endothelial cells are the major sites for PGI₂ production, the phenolic compounds in *Crataegus* extracts (which adsorbed on the nanosurface) may provide protection for endothelial cells by downregulating of pro-apoptotic gene expression levels which induce endothelial cell death (Ling et al., 2008). On the other hand, Garjani et al. (2000) reported that systolic arterial blood pressure decreases in rat when methanol extract is applied and this might be mediated by the protective action of PGI₂. Brixius et al. (2006) found that *Crataegus* extract induces vasorelaxation through endothelium-derived factors. Furthermore, isolated euscaphic acid from *C. azarolus* var. *aronia* L. can produce antivasoconstriction in rat aortic rings pre-incubated with indomethacin (Al-Habib et al., 2015). The antivasoconstriction effect of the CuO@Fe₃O₄@Walnut shell NCs used in the present study may be partly due to the presence of euscaphic acid in the NCs, because in the previous study, phytochemical screening results for the 1st time showed that *C. azarolus* var. *aronia* L. contains euscaphic acid.

One of the main constituents present in hawthorn extract is flavonoids (Shortle et al., 2014). Thus, the

Figure 4: Electron dispersive spectroscopy spectrum of green synthesized CuO@Fe₃O₄@Walnut shell nanocomposite (NCs), according to the quantitative elemental mapping, the presence of Fe, O, Cu, Al, and Mg confirms the anchoring of the CuO and magnetite nanocomposite norepinephrine on the surface of Walnut substrate and well synthesis of CuO@Fe₃O₄@Walnut shell NCs

Figure 5: Elemental mapping of the green synthesized CuO@Fe₃O₄@Walnut shell nanocomposite (NC), according to the qualitative elemental mapping, the presence of Al, Cu, Fe, Mg, and O confirms the well synthesis of CuO@Fe₃O₄@Walnut shell NCs

antivasoconstriction effect of CuO@Fe₃O₄@Walnut shell NCs may be partly due to the presence of flavonoids. Similarly, Wong (2004) indicated that hawthorn flavonoids induced vasorelaxation through the action of endothelium-derived relaxing factors and blockage the activity of

phosphodiesterase. Moreover, it was demonstrated that hawthorn extract can increase the cyclic guanosine monophosphate level which leads to vasodilation (Chen et al., 1998). Various K⁺ channels subtypes such as K_{Ca}, K_{ATP}, K_{IR}, and K_V channels are involved in the regulation of excitability of muscle fibers (Ko et al., 2008). Other studies recorded that (-)-epicatechin is an important bioactive hawthorn compound (Bahri-Sahloul et al., 2014). The data of the current study are consistent with previous researches that (-)-epicatechin enhances vasorelaxation action through the opening of K channels (Chen et al., 2000). The antivasoconstriction effect of CuO@Fe₃O₄@Walnut shell NCs may partly concern to the presence of (-)-epicatechin.

Antivasoconstriction Effects of CuO@Fe₃O₄@Walnut Shell NCs on Endothelium-intact Aorta

Dose response curves for CuO@Fe₃O₄@Walnut shell NCs antivasoconstriction effects on NE-induced contraction in aortic rings are shown in Figure 6. Green NCs (5*10⁻² mg/ml and 1*10⁻¹ mg/ml) caused significant antivasoconstriction effects ($P < 0.05$ and $P < 0.001$), respectively, on NE-induced dose-dependent contraction at 1*10⁻⁷–10⁻⁴ M in rat aortic smooth muscle rings as compared to the control rings.

The logarithm half maximal effective concentration (Log EC₅₀), Log EC₅₀ of CI 95%, and the maximum contraction for the antivasoconstriction effect of CuO@Fe₃O₄@Walnut shell NCs are shown in Table 1. CuO@Fe₃O₄@Walnut shell NCs at a concentration (5*10⁻² mg/ml) produced a significant anti-vasoconstriction effect on NE-induced contractions, with a Log EC₅₀ -8.065 mg/mL (Log EC₅₀ of CI 95% between -8.346 and -7.784), whereas the antivasoconstriction effect

of $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ shell NCs was increased by increasing the concentration to $1 \times 10^{-1} \text{ mg/ml}$ with $\text{Log EC}_{50} -8.376 \text{ mg/mL}$ (Log EC_{50} of CI 95% between -9.026 and -7.726). The Log EC_{50} was -8.300 mg/mL (Log EC_{50} of CI 95% between -9.163 and -7.437) in the absence of $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ shell NCs. Furthermore, $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ shell NCs at a concentration ($5 \times 10^{-2} \text{ mg/ml}$) also produced a significant antivasoconstriction effect on aortic rings since the maximum contraction was reduced from 1.635 ± 0.346 to 1.346 ± 0.084 . However, at a higher concentration ($1 \times 10^{-1} \text{ mg/ml}$), the maximum aortic contraction was further reduced to 0.095 ± 0.174 .

Antivasoconstriction Effects of $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ Shell NCs on Endothelium-denuded Aorta

Typical dose–response curves of NE in the absence and presence of $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ shell NCs ($5 \times 10^{-2} \text{ mg/ml}$ and $1 \times 10^{-1} \text{ mg/ml}$) in endothelium-denuded aortic rings are shown in Figure 7. Both doses of $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ shell NCs ($5 \times 10^{-2} \text{ mg/ml}$ and $1 \times 10^{-1} \text{ mg/ml}$) caused highly significant antivasoconstriction effects ($P < 0.001$) on NE-induced dose-dependent contraction at 1×10^{-9} – 10^{-4} M in endothelium-denuded aortic rings as compared to the control endothelium-denuded rat aortic rings (in the absence of $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ shell NCs).

The Log EC_{50} (Log EC_{50} of CI 95%) and the maximum contraction are shown in Table 2. Both NCs doses

($5 \times 10^{-2} \text{ mg/ml}$ and $1 \times 10^{-1} \text{ mg/ml}$) showed highly significant effects on NE contracted endothelium-denuded rat aortic rings with $\text{Log EC}_{50} -8.067 \text{ mg/mL}$ (Log EC_{50} of CI 95% between -9.230 and -6.904) and -6.521 mg/mL (Log EC_{50} of CI 95% between -6.770 and -6.271), respectively, whereas in the absence of $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ shell NCs, the Log EC_{50} was -10.46 mg/mL (Log EC_{50} of CI 95% between -38.11 and 17.19). Furthermore, $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ shell NCs ($5 \times 10^{-2} \text{ mg/ml}$) also showed a strong antivasoconstriction effect on endothelium-denuded aortic rings which was further enhanced at a higher dose ($1 \times 10^{-1} \text{ mg/ml}$), since the maximum contraction was reduced from 1.786 ± 0.116 in the control to 0.357 ± 0.090 and 0.144 ± 0.0001 , respectively.

Figure 6: Cumulative dose–response curves of norepinephrine in the absence and presence of $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ shell nanocomposite in endothelium-intact aortic rings

Table 1: The Log EC_{50} (Log EC_{50} of CI 95%) and maximum contraction for the effects of pre-incubation with $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ shell NCs on NE post-contracted endothelium-intact aortic rings

Vasoconstrictor Treatments	NE		
	Control	Green NCs $5 \times 10^{-2} \text{ mg/ml}$	Green NCs $1 \times 10^{-1} \text{ mg/ml}$
Log EC_{50}	-8.300	-8.065	-8.376
Log EC_{50} of CI 95%	-9.163– -7.437	-8.346– -7.784	-9.026– -7.726
Maximum contraction $\pm$ SEM	1.635 ± 0.346	1.346 ± 0.084	0.095 ± 0.174

Table 2: The Log EC_{50} (Log EC_{50} of CI 95%) and maximum contraction for the effects of pre-incubation with $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ shell NCs on NE post-contracted endothelium-denuded aortic rings

Vasoconstrictor Treatments	NE		
	Control	Green NCs $5 \times 10^{-2} \text{ mg/ml}$	Green NCs $1 \times 10^{-1} \text{ mg/ml}$
Log EC_{50}	-10.46	-8.067	-6.521
Log EC_{50} of CI 95%	-38.11–17.19	-9.230–6.904	-6.770–6.271
Maximum contraction $\pm$ SEM	1.786 ± 0.116	0.357 ± 0.090	0.144 ± 0.0001

Figure 7: Cumulative dose–response curves of norepinephrine in the absence and presence of $\text{CuO@Fe}_3\text{O}_4\text{@Walnut}$ shell nanocomposite in endothelium-denuded aortic rings

CONCLUSION

The results obtained by this study revealed that CuO@Fe₃O₄@Walnut shell NCs have antivasoconstriction and cardioprotective activities mediated possibly through enhancement of the production of endothelium-derived relaxing factors (particularly PGI₂). Furthermore, CuO@Fe₃O₄@Walnut shell NCs also increases the K⁺ conduction through increasing K⁺ channels activity. These investigations might be explaining the medicinal use of CuO@Fe₃O₄@Walnut shell NCs which green synthesized using *C. aronia* aqueous extract in CVDs as hypotensive agents. However, much more studies are required to establish the safety, efficacy, and activity of this NCs. Furthermore, due to the extreme antioxidant potential of the aqueous plant extract and also porous properties of walnut shell, they were used to the biosynthesis of bioactive CuO@Magnetite@Walnut shell NCs through an ecofriendly, fast, simple, and economic method which analyzing its structure using the micrographic techniques confirmed its nanosized structure.

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